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Microtubule mediation of cytoplasmic and nuclear maturation during the early stages of resumed meiosis in cultured mouse oocytes

(oocyte maturation/microtubule organizing centers/mitochondria)

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ABSTRACT **A perinuclear accumulation of mitochondria characterizes the premetaphase I stages of reinitiated meiosis in the laboratory mouse oocyte. The cellular basis of this organelle-specific translocation was examined by fluorescent probe analysis, immunostaining, and immunoelectron microscopy in oocytes cultured at specific stages of meiotic maturation in the presence and absence of drugs that influence the stability of microfilaments and microtubules. The results suggest that a temporal, spatial, and developmental relationship exists between the location of microtubule organizing centers and the progressive translocation of mitochondria to the nuclear region. The findings indicate that mitochondrial translocations are mediated by microtubules and that individual microtubule organizing centers are not only foci for mitochondrial aggregation but may also facilitate the establishment of the circular bivalent configuration.**

Preovulatory differentiation of the mammalian oocyte is a dynamic process that requires the coordinated expression of two distinct but interrelated developmental programs, nuclear and cytoplasmic maturation. Prior to germinal vesicle breakdown (GVBD), nuclear maturation involves changes in chromatin organization (1), nuclear membrane morphodynamics (2), and nucleolar function and stability (2, 3). After GVBD, nascent bivalent chromosomes form a circular configuration (circular bivalent stage; CBV) that subsequently develops into the first meiotic metaphase spindle. Nuclear maturation arrests at metaphase II after abstriction of the first polar body. Cytoplasmic maturation is associated with stage-related redistributions of organelles (4–7) and changes in transcriptional (8), translational (9), and posttranslational activities (10, 11). The normality of early mammalian embryogenesis is directly related to ordered expression of these developmental programs (12, 13).

In the laboratory mouse, the translocation and perinuclear accumulation of mitochondria is one aspect of the developmental program of cytoplasmic maturation observed between the GVBD and metaphase I (MI) stages (4). Our previous observations suggested that this accumulation is mediated by microtubules (2, 4, 14, 15) and may be required for nuclear maturation to progress after MI. The present findings support these notions and provide evidence that mitochondrial translocations are temporally and spatially coordinated with changes in the location of microtubule organizing centers (MTOCs). The results also suggest that the formation of the CBV configuration may be influenced by the presence and spatial distribution of perinuclear MTOCs. Some of the results were previously reported in a preliminary form (2).

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MATERIALS AND METHODS

Oocyte Collection and Culture and Fluorescent Probe Analysis. Full-grown oocytes with a centrally located germinal vesicle (GV) were harvested from the antral follicles of 6- to 8-week-old heterogeneous stock (HS) mice in medium M2 containing N^6,$O^{2'}$-dibutyryladenosine 3',5'-cyclic monophosphate (Bt_2cAMP) at 100 μg/ml. Oocytes were denuded of adherent granulosa cells by repeated passage through a micropipet (4). After collection, GV-stage oocytes were cultured in groups of 60 in 100-μl microdrops of Bt_2cAMP-free M2 medium in the presence or absence of either taxol (2), nocodozole (14), or cytochalasin B (14, 16), with each inhibitor used at a concentration of 5 μg/ml. After an initial culture in M2, oocytes were exposed to these inhibitors at 15-min intervals during the GV stage and at 30-min intervals during the GVBD, CBV, and early MI stages of maturation. Living oocytes were examined by differential interference contrast microscopy, followed by a determination of mitochondrial distributions and chromosomal organization with the fluorescent probes rhodamine 123 and 4',6-diamidino-2-phenylindole, respectively (4, 15). Under the following conditions and as part of ongoing studies of meiotic maturation, 2735 oocytes have been analyzed at the indicated stages of maturation by differential interference contrast microscopy and fluorescent microscopy: (*i*) 700 untreated; (*ii*) 875 nocodozole-exposed; (*iii*) 650 taxol-treated; (*iv*) 510 cytochalasin B-exposed. In this study, 200 oocytes from each group were prepared for anti-tubulin immunostaining, and of these, 68 normally matured, 60 taxol-treated, 38 nocodozole-treated, and 41 cytochalasin B-exposed oocytes were then examined by transmission electron microscopy (TEM).

Immunocytological Staining. Oocytes were denuded of the zona pellucida by exposure to acid Tyrode's solution (17), washed in PHEM buffer (18) supplemented with 5% bovine serum albumin, and fixed in PHEM buffer containing 1% paraformaldehyde for 10 min. After fixation, oocytes were extracted for 10–30 min in a buffer containing 25% glycerol, 10 mM 2-mercaptoethanol, 25 mM Hepes, 0.5 mM $MgCl_2$, 10 mM EGTA, 25 μM phenylmethylsulfonyl fluoride, and 0.3% Triton X-100. For overnight extraction, a buffer containing 0.1 M Pipes, 2.5 mM EGTA, 2.5 M $MgCl_2$, 1 M hexylene glycol, and 0.1% Triton X-100 (pH 6.9 at 5°C) was used. Fixed oocytes were exposed to a mouse anti-tubulin monoclonal antibody for 30–60 min followed by exposure either to fluorescein-labeled anti-mouse immunoglobulin antibodies as described (15) or to a goat anti-mouse antibody conjugated to horseradish peroxidase. For the latter protocol, anti-tubulin staining was amplified by incubation in a solution containing 3,3'-diaminobenzidine (0.4 mg/ml), $NiCl_2$ (0.3 mg/ml), and

Abbreviations: GV, germinal vesicle; GVBD, germinal vesicle breakdown; CBV, circular bivalent stage; MI, metaphase I; MTOC, microtubule organizing center; TEM, transmission electron microscopy.

0.01% H_2O_2 (from a 30% stock solution). For light and fluorescence microscopy, oocytes were placed on poly(L-lysine)-coated glass coverslips in phosphate-buffered saline containing 5% propyl gallate and examined by epifluorescence illumination. Antibody-treated oocytes were prepared for TEM by conventional methods (4, 15).

RESULTS

Mitochondrial Translocations and the Spatial Organization of Microtubules. Optical sections of newly harvested, anti-tubulin-stained GV-stage oocytes detected between 10 and 14 discrete foci of tubulin in the pericortical cytoplasm (Fig. 1 *a–c*). Consecutive thin sections analyzed by TEM demonstrated that the tubulin foci were MTOCs and that microtubules that emanated from adjacent MTOCs were frequently interdigitated (Fig. 1*d*). After extraction and immunostaining, MTOCs appeared to have a ring-like core (Fig. 1 *d* and *i*). Approximately 30–45 min after the initiation of culture, the following cytoplasmic changes were observed: (*i*) in contrast to the uniform distribution of mitochondria characteristic of early GV stage (Fig. 1*e*), small clusters of mitochondria formed in the pericortical cytoplasm (Fig. 1*f*), (*ii*) MTOCs were now located in deeper portions of the cytoplasm (Fig. 1*g*), and (*iii*) interdigitations of microtubules from adjacent MTOCs were no longer apparent. In 79% (55/70) of the oocytes examined between 45 and 60 min of culture, all detectable MTOCs were distributed around the periphery of GV (Fig. 1*h*). For the other 21%, no perinuclear accumulation of MTOCs was observed. In this respect, anti-tubulin immunostaining of oocytes that failed to resume meiosis or that arrested meiosis at GVBD showed either few (one to five) or no perinuclear MTOCs (2). Immediately preceding GVBD (1.0–1.5 hr), MTOCs were juxtaposed with the nuclear envelope (Fig. 1*h*). During the GVBD-to-CBV transition (Fig. 1*k*), the perinuclear cytoplasm contained small arrays of microtubules that originated from GV-associated MTOCs (Fig. 1 *i* and *l*). As reported previously (4), the cortical cytoplasm was progressively depleted of mitochondria as these organelles moved centrally (Fig. 1*j*). At early MI, the nuclear region was not only surrounded by a dense accumulation of mitochondria (Fig. 1 *m* and *n*) but also exhibited intense α-tubulin staining (Fig. 1*o*). Consequently, individual perinuclear MTOCs and arrays of microtubules were extremely difficult to detect at this stage of maturation.

Aberrant Patterns of Mitochondrial Organization During Normal Maturation and After Exposure to Microtubule Inhibitors. Approximately 6% of untreated CBV-to-MI stage oocytes displayed both perinuclear mitochondria and a variable number (one to eight) of small mitochondrial aggregates not associated with the nuclear region. These aggregates were usually present in groups of two or three (Fig. 2 *a*, *e*, and *g*) and were most often confined to the same region of the cortical cytoplasm (Fig. 2 *a*, *b*, *e*, and *g*). For intact oocytes, TEM revealed the presence of a ring-like structure within each aggregate (Fig. 2 *b* and *c*). Optical and serial thin

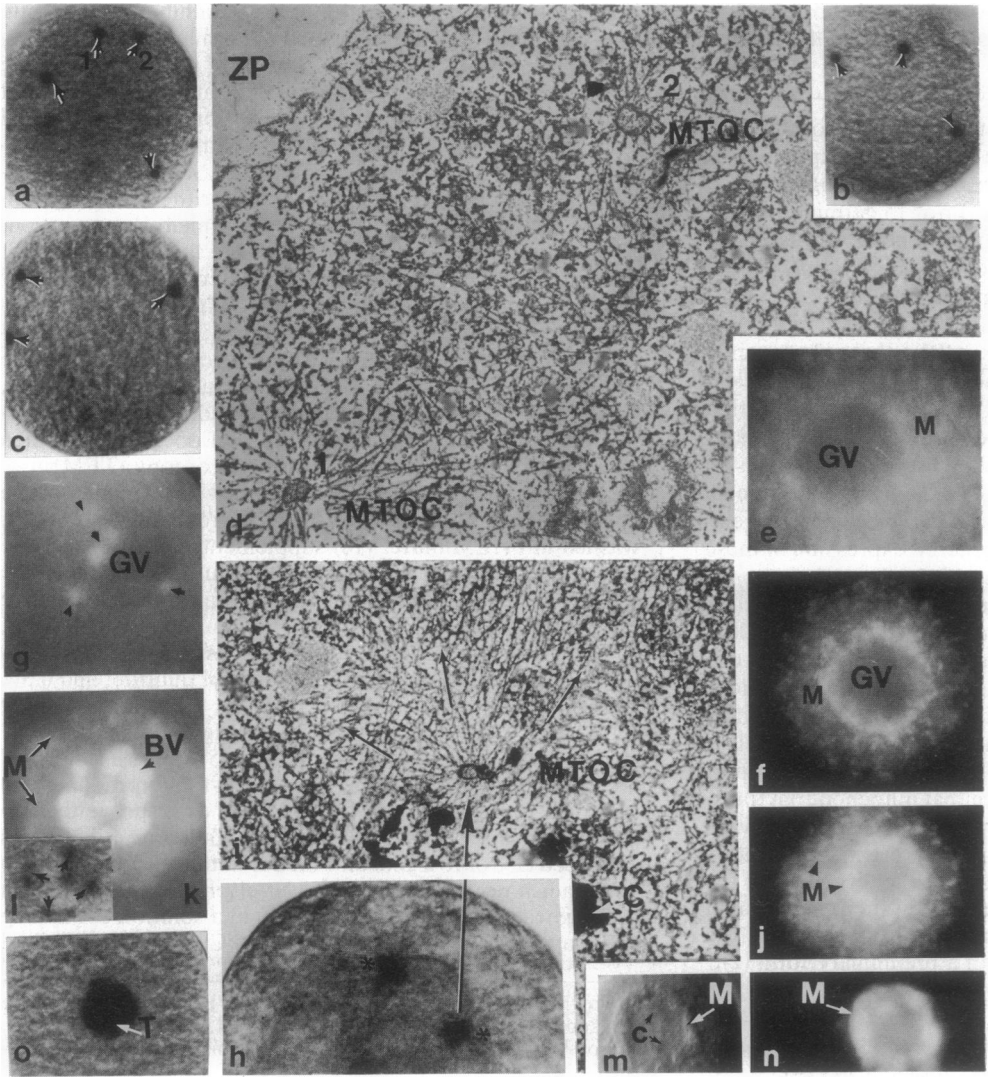

FIG. 1. The cortical distribution of discrete tubulin foci (arrows) in a newly harvested GV-stage oocyte is shown in *a–c* (anti-tubulin immunostaining with 3,3′-diaminobenzidine enhancement). The TEM image of this oocyte, shown in *d*, demonstrates that the tubulin foci (e.g., indicated by arrows 1 and 2 in *a*) are MTOCs. (*g*) Distribution of MTOCs (indirect immunofluorescence) at the periphery of the GV typically observed by 45 min of maturation. Approximately 60 min after the resumption of meiosis, MTOCs were adjacent to the nuclear envelope (asterisks in *h*). (*i*) Both the TEM image of the 3,3′-diaminobenzidine enhanced, immunostained MTOC (adjacent to the arrow in *h*) and the characteristic cytoplasmic orientation of perinuclear microtubules (arrows in *i*) are shown. The progressive perinuclear accumulation of mitochondria (M) in a rhodamine 123-stained oocyte observed at 0 min, 60 min, and 2.5 hr is shown in *e*, *f*, and *j*, respectively. Staining this oocyte in the living state at 2.5 hr with 4′,6-diamidino-2-phenylindole (*k*) and after fixation with anti-tubulin demonstrates the circular bivalent configuration (BV) and the perinuclear distribution of MTOCs (arrows in *l*), respectively. By early MI, the perinuclear accumulation of mitochondria is complete (*m* and *n*) and the chromosomal region (c) (*m*) enclosed by mitochondria (M in *n*) stains intensely with anti-tubulin (T) (*o*). (*d*, ×2470; *i*, ×1950.) ZP, zona pellucida.

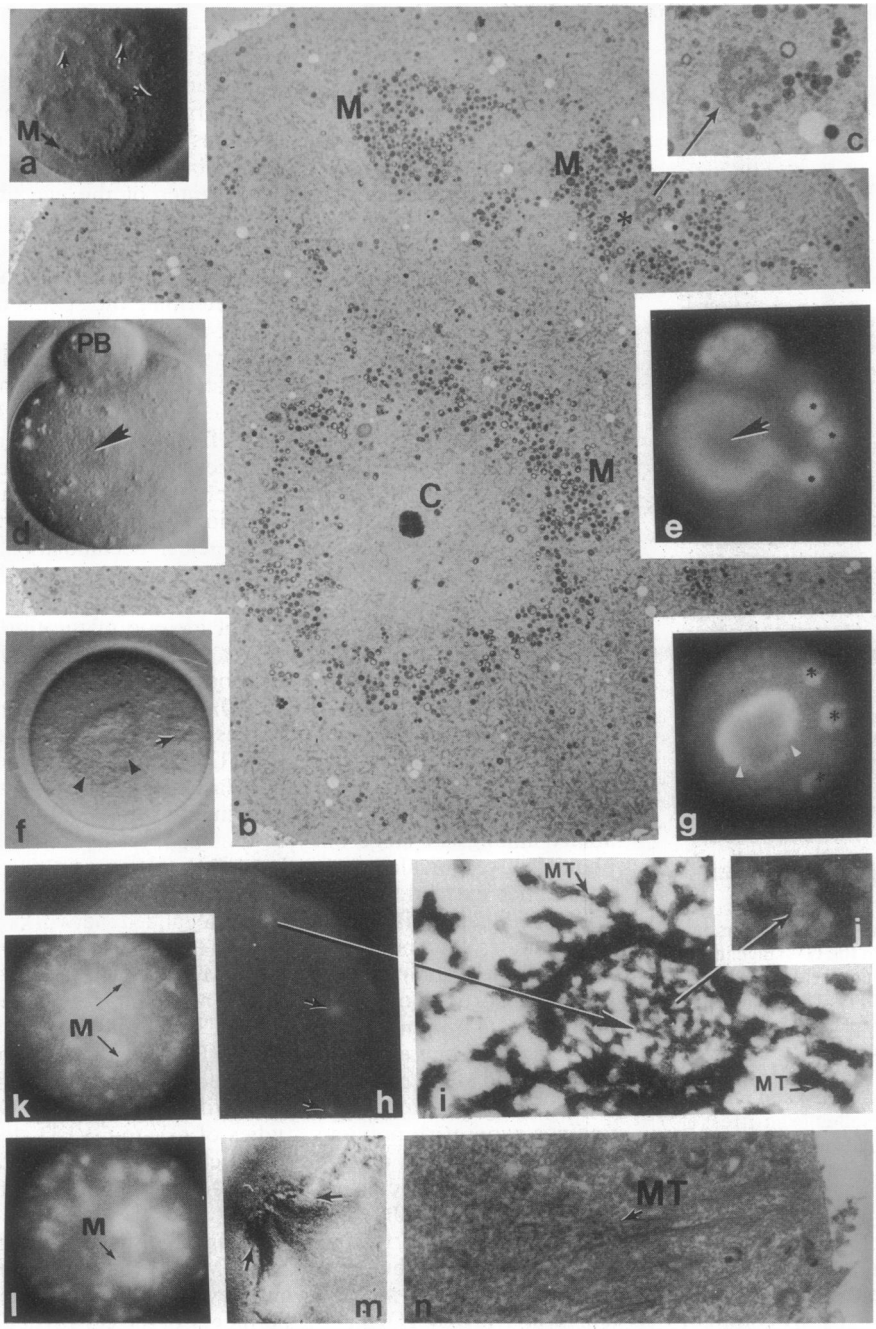

FIG. 2. Differential interference contrast microscopy (*a*) and TEM (*b* and *c*) images of an oocyte that failed to form the MI spindle and that contained both perinuclear (M) and cytoplasmic (arrows) accumulations of mitochondria (*a*). TEM analysis of this oocyte demonstrated the presence of a ring-like structure (asterisk in *b*) within each cytoplasmic aggregate (arrow in *c*). Abstriction of the first polar body (PB) (*d*) usually occurred in oocytes containing up to four MTOC/mitochondrial complexes that were identified by rhodamine 123 fluorescence (asterisks in *e*). Oocytes exposed to taxol between 15 and 30 min after resumption of meiosis frequently developed multiple mitochondrial aggregates in the cortical cytoplasm (one of which is indicated by an arrow in *f*, and three by asterisks in *g*) and a perinuclear mitochondrial accumulation that was incomplete and of reduced fluorescence (arrowheads in *f* and *g*). Immunostaining and TEM of this representative oocyte demonstrated the presence of tubulin (arrows in *h*) and of an MTOC (*i*) within each mitochondrial aggregate. At higher magnification, microtubules (MT) are evident in the ring-like core of the MTOC (*j*). No perinuclear accumulation of mitochondria occurred in oocytes exposed to nocodozole (*k*) or taxol (*l*) from the GV stage. However, with continued exposure to taxol, thick bundles of subplasmalemmal microtubules (arrows in *m*) extended into the cortical cytoplasm (*n*). (*b*, ×1400; *c*, ×8400; *i*, ×18,200; *j*, ×35,000; *n*, ×7000.)

sections of rhodamine 123/α-tubulin-stained oocytes demonstrated that mitochondria (asterisks in Fig. 2*g*) were organized in a spherical shell around a tubulin-rich interior (arrows in Fig. 2*h*). By TEM the interior of each aggregate was found to contain the ring-like core and the associated microtubules of an MTOC (Fig. 2 *i* and *j*). The normal progression of chromosomal maturation and polar body abstriction usually occurred in oocytes containing up to four cytoplasmic MTOC/mitochondrial complexes (Fig. 2 *d* and *e*). Typically, the presence of six or more cytoplasmic MTOC/mitochondrial complexes was associated with (*i*) an arrest of meiosis prior to MI and (*ii*) a nonuniform perinuclear accumulation of mitochondria (arrowheads in Fig. 2 *f* and *g*) that, in comparison to normally maturing oocytes (Fig. 1 *m* and *n*), exhibited a reduced intensity of rhodamine 123 fluorescence (Fig. 2*g*).

Eighty-seven percent of the GV-stage oocytes cultured in the presence of cytochalasin B resumed meiosis, translocated mitochondria to the perinuclear region (14), and underwent apparently normal chromosomal maturation to MI but did not abstrict the first polar body (14, 16). In contrast, >85% of newly harvested GV-stage oocytes (i.e., 0 min) cultured in medium containing either nocodozole or taxol underwent GVBD, but the CBV configuration did not develop and no perinuclear accumulation of mitochondria was observed (Fig. 2 *k* and *l*). For oocytes exposed to *nocodozole* from the GV stage on, neither cytoplasmic arrays of microtubules nor MTOCs were detected by immunostaining and TEM. However, after approximately 3 hr of culture in the presence of *taxol*, bundles of microtubules extended into the pericortical cytoplasm from a subplasmalemmal origin (Fig. 2 *m* and *n*). The number of discrete bundles of microtubules varied among oocytes from 7 to 18. By 8 hr, relatively large aggregates of mitochondria (Fig. 3*b*) had developed in close proximity to these asters (Fig. 3*a*).

Approximately 70% of the oocytes transferred to taxol-containing medium 30 min after the reinitiation of meiosis developed both a perinuclear accumulation of mitochondria

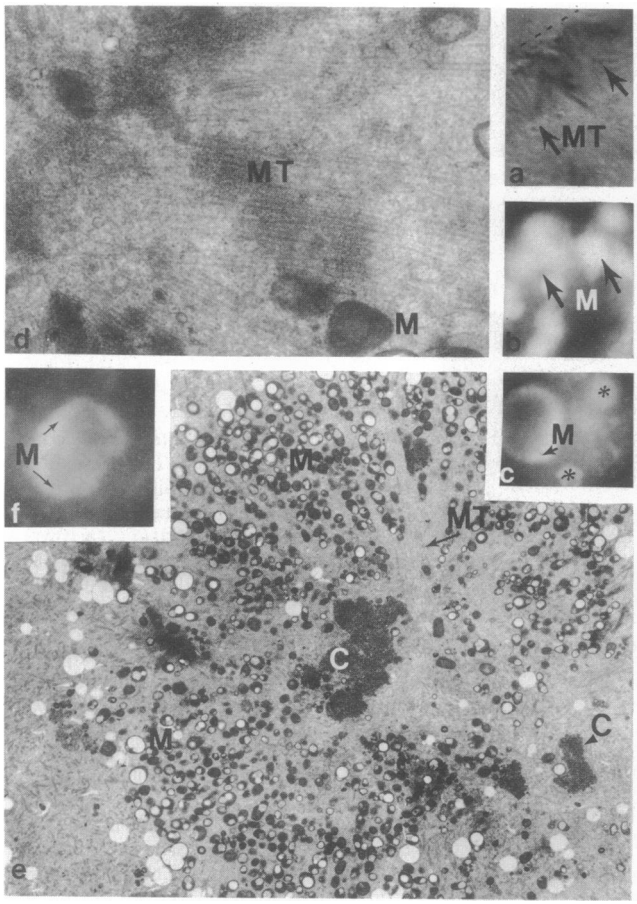

FIG. 3. Rhodamine 123 fluorescence (*b*) and anti-tubulin immunostaining (*a*) of an oocyte cultured for 5 hr from the GV stage in the presence of taxol. Large aggregates of mitochondria (M) (*b*) formed in proximity to these bundles of microtubules (MT). The dashed line in *a* denotes the approximate position of the plasma membrane. Oocytes exposed to taxol 30–40 min after the resumption of meiosis frequently exhibited both perinuclear mitochondria (M) (*c*) and cytoplasmic MTOC/mitochondrial complexes (asterisks in *c*). Culture of GVBD-to-CBV stage oocytes in taxol resulted in an arrest of meiosis and the formation of thick bundles of microtubules (*d*) that penetrated the perinuclear shell of mitochondria (*e* and *f*). C, bivalent chromosomes. (*d*, ×15,600; *e*, ×3000.)

and a variable number (two to eight) of pericortical MTOC/mitochondrial complexes (asterisks in Fig. 3*c*). An apparently normal CBV configuration was present in oocytes with as many as four of these cytoplasmic complexes. By contrast, the CBV configuration was either incomplete, or more typically, did not develop when six or more cytoplasmic mitochondrial aggregates were detected by rhodamine 123 staining (data not shown). Although nuclear maturation arrested at the CBV configuration in oocytes exposed to taxol during the GVBD (1.0–1.5 hr) or CBV (2–3 hr) stages, the perinuclear accumulation of mitochondria continued. Approximately 6 hr after exposure of GVBD-to-CBV stage oocytes to taxol, thick bundles of microtubules (Fig. 3*d*) penetrated the enveloping perinuclear mitochondria. After 10 hr of taxol exposure, the CBV configuration was no longer evident, individual chromosomes were observed to migrate away from the nuclear region (Fig. 3*e*), and the arrays of perinuclear microtubules appeared to have increased in thickness and length (arrow in Fig. 3*e*). The perinuclear mitochondrial shell persisted for at least an additional 12 hr of culture (Fig. 3*f*). Although meiotic maturation arrested at the CBV stage, no cytoplasmic MTOC/mitochondrial complexes were observed in the other 30% of the oocytes exposed to taxol 30 min after the spontaneous reinitiation of meiosis.

DISCUSSION

Developmental events of mouse oocyte maturation and early embryogenesis that may require the participation of MTOC-associated microtubules include (*i*) formation of metaphase spindles during the two meiotic divisions of the oocyte and the first four mitotic divisions of the embryo (19, 20) and (*ii*) migration of pronuclei toward the center of the egg after fertilization (20). In contrast, whether MTOCs participate in the *cytoplasmic* maturation of the oocyte has not been determined. However, when it is considered that MTOCs organize networks of microtubules that can determine the spatial distribution of organelles (21), including mitochondria (22), the present findings suggest that a similar subcellular mechanism may mediate the perinuclear translocation of mitochondria in the mouse oocyte (2, 4, 14–16). Indirect evidence for such a role was previously indicated by the finding that the perinuclear accumulation of mitochondria occurred in the absence of microfilaments (14, 15) but not in the absence of microtubules (2, 14). Indeed, very similar observations have been reported for the rat oocyte, where the perinuclear accumulation of lysosome-like organelles that normally occurs prior to GVBD is inhibited by nocodozole (5).

Our findings indicate that onset of meiotic maturation in the mouse oocyte is accompanied by a rapid inward migration of MTOCs. The full-grown mouse oocyte has been reported to contain between 8 (16) and 16 (20) MTOCs. With immunostaining and TEM of newly harvested GV-stage oocytes, we detected between 10 and 14 MTOCs, all of which were initially located in the cortical cytoplasm. The inconsistency in MTOC number may reflect an actual developmental variability among GV-stage oocytes that appeared to be morphologically equivalent at retrieval (16), or may be due to differential extraction or inadequate antibody binding, or both. TEM analysis indicated that localized networks of microtubules exist between cortical MTOCs. However, ≈30 min after the resumption of meiosis, MTOCs were observed in deeper portions of the cytoplasm, and contacts between microtubules from neighboring MTOCs were no longer evident. In most oocytes examined at GVBD, all of the MTOCs were located at the periphery of the nuclear membrane, with microtubules directed primarily towards the cytoplasm. Our findings agree with previous studies of GV-to-GVBD stage mouse oocytes that detected perinuclear MTOCs and described a cytoplasmic displacement of the associated microtubules (2, 16, 19, 20).

We interpret the following findings as suggesting that changes in the distribution of oocyte MTOCs and mitochondria are temporally, spatially, and developmentally related: (*i*) The arrival of MTOCs at the periphery of the GV *preceded* any significant perinuclear translocation of mitochondria. (*ii*) During translocation, small mitochondrial clusters were located between slender arrays of microtubules that emanated from perinuclear MTOCs (2). (*iii*) No perinuclear translocation of mitochondria occurred in GV-stage oocytes exposed to nocodozole, which rapidly induces the depolymerization of microtubules (15). (*iv*) No inward movement of MTOCs or perinuclear translocation of mitochondria was observed in GV-stage oocytes harvested and cultured in the presence of taxol. However, exposure of these oocytes to taxol, which promotes the assembly of microtubules, was characterized by the formation of bundles of microtubules that extended into the cortical cytoplasm from subplasmalemmal locations and by the absence of any detectable perinuclear microtubules. The number of cortical bundles was within the same range as the number of MTOCs detected in untreated GV-stage oocytes. This finding suggests that taxol promoted the growth of microtubules from MTOCs that, in the presence of this drug, remained in the cytocortex. With continued culture, large aggregates of mitochondria formed in close prox-

imity to the bundles of microtubules. (*v*) GV-stage oocytes exposed to taxol after 30 min of culture frequently exhibited multiple cytoplasmic mitochondrial/MTOC complexes and a perinuclear accumulation of mitochondria that was of a reduced density. The inward movement of MTOCs appears to arrest in oocytes exposed to taxol at the very early stages of maturation. However, the presence of cytoplasmic MTOC/mitochondrial complexes in both untreated and taxol-exposed oocytes demonstrates that individual MTOCs are foci for mitochondrial aggregation. (*vi*) The perinuclear accumulation of mitochondria continued in oocytes exposed to taxol at GVBD. These oocytes subsequently developed thick bundles of microtubules that passed through the perinuclear mitochondrial layer. Individual cytoplasmic MTOC/mitochondrial complexes were infrequently observed in oocytes exposed to taxol at the GVBD and CBV stages. This finding is consistent with the observation that for most oocytes, MTOCs were already in a perinuclear location when taxol was added. Our observations support the findings of Rime *et al.* (23), who described the presence of large arrays of microtubules at the periphery of the nucleus after exposure of GV-stage mouse oocytes to taxol.

It has previously been suggested that the ability of the cytoplasm to translocate mitochondria may have evolved in the laboratory mouse to concentrate the limited energy-producing capacity of the oocyte to specific cytoplasmic regions that need a higher level of ATP than would otherwise be available to support such developmental processes as spindle assembly, disassembly, chromosomal movement, and polar body formation (4, 14). A significant reduction in the apparent density of perinuclear mitochondria occurred in oocytes with multiple pericortical mitochondrial/MTOC complexes. Although these oocytes underwent GVBD and chromosomal condensation, they rarely formed a MI spindle. Although it is tempting to conclude that the progression of meiotic maturation is dependent upon the presence of a critical density of perinuclear mitochondria, it is also possible that meiotic arrest is associated with a reduction in the number of perinuclear MTOCs available to form the poles of the metaphase spindles (16, 19, 20). Maro *et al.* (20) reported that the mouse oocyte contains up to 16 MTOCs, 3 of which are located at each spindle pole. However, the presence of multiple cytoplasmic MTOC/mitochondrial complexes in oocytes that also displayed 6 or more perinuclear MTOCs suggests that a deficiency of nuclear envelope-associated MTOCs may not be the primary cause of meiotic arrest. We tentatively propose that meiotic arrest in these oocytes is due to an energy deficiency rather than MTOC deficiency.

The present results also suggest that MTOCs provide a specific perinuclear geometry associated with the establishment of the CBV configuration. Prior to GVBD, microtubules from perinuclear MTOCs contact condensing chromatin through small breaks in the nuclear envelope (19). Although GV-stage oocytes exposed to nocodozole or taxol upon removal from antral follicles undergo GVBD, the CBV stage does not develop and the nascent bivalent chromosomes aggregate to form a compact mass in the center of the oocyte (15). The failure of the CBV configuration to develop in these oocytes may be a consequence of the absence of perinuclear MTOCs. In this respect, in the presence of six or more cytoplasmic mitochondrial/MTOC complexes, the CBV configuration failed to form or displayed a perturbed organization. In contrast, the prolongation of the CBV stage in oocytes exposed to taxol at or shortly after GVBD may involve a persistent rather than transient interaction between the nascent bivalents and microtubules from perinuclear MTOCs.

The cellular and molecular conditions responsible for the movement of MTOCs at the outset of resumed meiosis in the mouse oocyte are unknown (2). However, changes in the state of phosphorylation of MTOC-associated proteins are accompanied by a modulation of the microtubule-organizing capacity of these organelles in mitotic cells (24). In this respect, significant quantitative and qualitative changes in the pattern of protein phosphorylation are detected in the mouse oocyte minutes after the spontaneous resumption of meiosis *in vitro* (10, 25, 26). The extent to which, if any, the inward movement of pericortical MTOCs is associated with or dependent upon these posttranslational modifications is currently under investigation.

The dynamics of microtubule-mediated organelle movement is well-established for such processes as axoplasmic transport (27), extension of endoplasmic reticulum tubules (28), pigment granule translocation (21), and changes in mitochondrial distribution (22). Early work by Heggeness *et al.* (22) suggested that the microtubule-mediated regulation of mitochondrial distribution is a phenomenon of general physiological significance that (*i*) involves a specific linkage between these two cellular components and (*ii*) may be important in controlling local concentrations of ATP, divalent cations, and other cell constituents involved in mitochondrial metabolism. Taken together, our present and previous observations (2, 4, 15) support the notion that microtubules mediate the preinuclear accumulation of mitochondria during the maturation of the mouse oocyte *in vitro* in order to provide locally high levels of ATP that may be necessary for nuclear maturation (chromosomal) after the CBV stage. Whether the mechanism of mitochondrial translocation in the mouse oocyte involves transport along individual microtubules, or movement within channels created by arrays of perinuclear microtubules, or both is unknown (2).

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